12-0 Rec'd PCT/PTO 05 DEC 2000

FORM PTO-1390 (Modified) (REV 11-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

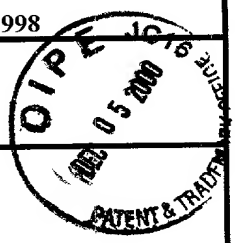
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 22727/04080

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR): 09/719024

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US99/12680	04 June 1999	05 June 1998

TITLE OF INVENTION
MUTANT FORMS OF THE AL2 GENE OF GEMINIVIRUSES



APPLICANT(S) FOR DO/EO/US
Bisaro, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

Copy of Declaration and Power of Attorney
Copy of Small Entity Statement (Non-Proft Organization)
Return Receipt Postcard

PCTUS1/REV03

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U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/719024	PCT/US99/12680	22727/04080

21. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1,000.00**	
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but Internation Search Report prepared by the EPO or JPO **$860.00**	
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**	
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**	
☒	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
	ENTER APPROPRIATE BASIC FEE AMOUNT =	**$100.00**
	Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	24 - 20 =	4	x $18.00	**$72.00**	
Independent claims	4 - 3 =	1	x $80.00	**$80.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
	TOTAL OF ABOVE CALCULATIONS =			**$252.00**	
	Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable).** ☒			**$126.00**	
	SUBTOTAL =			**$126.00**	
	Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +			**$0.00**	
	TOTAL NATIONAL FEE =			**$126.00**	
	Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐			**$0.00**	
	TOTAL FEES ENCLOSED =			**$126.00**	
				Amount to be: refunded	$
				charged	$

☒ A check in the amount of **$126.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **03-0172** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

Brian D. Johnson
NAME

40,665
REGISTRATION NUMBER

Dec. 5, 2000
DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF	:	Bisaro
FOR	:	**MUTANT FORMS OF THE AL2 GENE OF GEMINIVIRUSES**
SERIAL NO.	:	09/092,705
FILED	:	June 5, 1998
ATTORNEY DOCKET NO.	:	22727/04003

37 C.F.R. 1.27 VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS-NONPROFIT ORGANIZATION

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION:	Ohio State Research Foundation
ADDRESS:	1960 Kenny Road Columbus, Ohio 43210-1063

TYPE OF ORGANIZATION

- ☐ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
- ☒ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))
- ☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
  (name of state _____)
  (citation of statute _____)
- ☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
- ☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
  (name of state _____)

(citation of statute _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled:

MUTANT FORMS OF THE AL2 GENE OF GEMINIVIRUSES

by inventors: David M. Bisaro

described in

- ☐ the specification filed herewith.
- ☒ application Serial No. 09/092,705; Filed: June 5, 1998.
- ☐ Patent No. _____,
- ☐ issued _____.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention. If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

* Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING **David N. Allen, Ph. D.**

TITLE IN ORGANIZATION Director, Technology Transfer

ADDRESS OF PERSON SIGNING 1960 Kenny Road
Columbus, Ohio 43210-1063

SIGNATURE David N. Allen DATE 13 July '98

Mutant Forms of the AL2 Gene of Geminiviruses

Background of the Invention

The Geminiviridae is a family of single-stranded DNA plant viruses. The geminiviruses in the genus Begomovirus (formerly known as subgroup III) are transmitted to plants by whiteflies. The geminiviruses in the genus Begomovirus infect most dicots including, for example, vegetable crops such as beans, squash, pepper, tomato, cassava, and potato; as well as tobacco, and fiber crops such as cotton and kenaf. Such infection can result in total loss of the infected crop.

The frequency of geminivirus epidemics in susceptible plants has increased in recent years, most likely due to an increase in whitefly populations which are resistant to insecticides. Accordingly, it is desirable to have techniques to combat geminiviruses.

Summary Of The Invention

The present invention provides isolated double domain mutant AL2 genes of a geminivirus. The double domain mutant AL2 gene is useful for producing transgenic plants that are more resistant to infection by geminiviruses, particularly Begomoviruses. The double domain AL2 mutant gene comprises at least one mutation in the 3' region of the AL2 gene, i.e., the region which encodes about 46 amino acids extending from about amino acid 83 to about amino acid 129 of the wild-type AL2 gene product and at least one mutation in the central region of the AL2 gene , i.e., the region which encodes about 20 amino acids extending from about amino acid 23 to about amino acid 43 of the wild-type AL2 gene product

The present invention also provides isolated single domain mutant AL2 genes of a geminivirus. The single domain mutant AL2 genes are useful for producing transgenic plants that are more susceptible to infection by geminiviruses, particularly Curtoviruses (formerly known as subgroup II) and Begomoviruses. The single domain mutant AL2 gene comprises at least one mutation in the 3' region of the AL2 gene, the region which encodes about 46 amino acids extending from about amino acid 83 to about amino acid 129 of the wild-type AL2 gene product.

The present invention also relates to vectors comprising a double domain mutant AL2 gene and to vectors comprising a single domain mutant AL2 gene. The present invention also relates to transgenic plants comprising a double domain mutant AL2 gene and to transgenic plants comprising a single domain mutant AL2 gene.

Brief Description of the Figures

Fig. 1 shows the amino acid sequence, SEQ ID NO:1, of the AL2 gene product, hereinafter referred to as the "transcriptional activator protein" or "TrAP", of tomato golden mosaic virus (TGMV)(see arrow). The amino acid sequence of TMGV TrAP is aligned with the amino acid sequences of the TrAP proteins of Abutilon mosaic virus (AbMV), bean dwarf mosaic virus (BDMV), Bean golden mosaic virus (BGMV), cabbage leaf curl virus (CabLCV), potato yellow mosaic virus (PYMV), sida golden mosaic virus (SGMV), squash leaf curl virus (SqLCV), tomato mottle virus (ToMV), and Texas pepper virus (TPV).

Fig. 2 shows the 5' primers and 3' primers used to construct substitution mutants of the 3' region of the TGMV AL2 gene.

Fig. 3 is a graph depicting the extent of transcriptional activation by fusion proteins comprising a GAL 4 binding domain fused to a full-length TGMV TrAP protein or fragments thereof.

Fig. 4 is a graph depicting the extent of transcriptional activation by fusion proteins comprising a GAL 4 binding domain fused to fragments of TGMV TrAP, which fragments do and do not contain amino acid substitutions in the wild-type sequence.

Detailed Description Of The Invention

The AL2 genes of the whitefly-transmitted geminiviruses encode a protein, referred to hereinafter as "TrAP". TrAP is a transcriptional activator that is required for expression of the genes involved in infectivity, systemic spread, and insect transmission of such geminiviruses. Specifically, TrAP is a transcriptional activator that controls expression of the late genes that encode the capsid protein and a movement protein, CP and BRI, respectively.

TrAP is a small protein of approximately 15 kDa. Begomovirus TrAP sequences are highly conserved. The amino acid sequence of the TrAP protein of TGMV is shown in Fig. 1. TrAP has a modular structure which comprises an amino terminal basic domain, a central region, and a carboxy terminal acidic domain. The central region of TrAP is characterized by a series of strictly conserved cysteine and histidine residues. These conserved residues are arranged as $C\text{-}X_1\text{-}C\text{-}X_4\text{-}H\text{-}X_2\text{-}C\text{-}X_6\text{-}H\text{-}X_4\text{-}HC$ in TrAP from all Begomoviruses except squash leaf curl virus (SqLCV), which has a slightly different spacing: $C\text{-}X_1\text{-}C\text{-}X_4\text{-}H\text{-}X_2\text{-}C\text{-}X_8\text{-}H\text{-}X_4\text{-}HC$, wherein the X subscript indicates the number of amino acids separating the cysteine and histidine residues.

Mutant AL2 Genes

In accordance with the present invention, mutant forms of the AL2 gene of a geminivirus are provided. The nucleotide sequences of the wild-type, i.e., non-mutant forms of the AL2 genes of the geminiviruses are readily available in GenBank. The accession numbers for geminiviruses whose genomes contain an AL2 gene are as follows:

Table 1

Accession Numbers for geminivirus DNA sequences containing the AL2 gene (also known as AC2 or C2), which encodes TrAP.

Virus	Accession Number
Abutilon mosiac virus Hawaii strain	U51137
Abutilon mosaic virus West Indies isolate	X15983
African cassava mosaic virus West Kenyan isolate	J02057
African cassava mosaic virus Nigerian isolate	X17095
Ageratum yellow vein virus	X74516
Althea rose enation virus Egypt isolate	AF014881
Angled luffa leaf curl virus Thailand isolate	AF102276/AF061339
Bean dwarf mosaic virus	M88179
Bean golden mosaic virus Puerto Rico isolate	M10070
Bean golden mosaic virus Brazil isolate	M88686

Table 1 (con't)

Bean golden mosaic virus Dominican Republic isolate	L01635
Bean golden mosaic virus Guatemala isolate	M91604
Cabbage leaf curl virus	U65529
Chayote mosaic virus Ibadan (Nigeria) isolate	AJ223191
Cowpea golden mosaic virus Nsukka (Nigeria) isolate	AF029217
Cotton leaf curl virus Pakistan isolate (clc62)	AJ002447
Cotton leaf curl virus Pakistan isolate (clc72b)	AJ002448
Cotton leaf curl virus Pakistan isolate (clc806b)	AJ002449
Cotton leaf curl virus Pakistan isolate (clc804a)	AJ02452
Cotton leaf curl virus Pakistan isolate (clc802a)	AJ002455
Cotton leaf curl virus Pakistan isolate (clc26)	AJ002458
Cotton leaf curl virus Pakistan okra leaf curl isolate (olc311)	AJ002459
Havana tomato virus Cuba isolate	Y14874
Indian cassava mosaic virus	Z24758
Indian mungbean yellow mosaic virus Isolate bg3	AF126406
Ipomoea yellow vein virus Spain isolate	AJ132548
Mungbean yellow mosaic virus Japan isolate	D14703

Table 1 (con't)

Mungbean yellow mosaic virus Thailand isolate	AB017341
Okra yellow vein virus Pakistan isolate (oyvm201)	AJ002451
Okra yellow vein virus Pakistan isolate (oyvm301)	AJ002453
Papaya yellow mosaic virus	Y15934
Pepper hausteco virus Mexico isolate	X70418
Potato yellow mosaic virus	D00940
Sida golden mosaic virus Costa Rica isolate	X99550
Sida golden mosaic virus Honduras isolate	Y11097
Sida golden mosaic virus Honduras isolate (yellow vein)	Y11099
Sida golden mosaic virus Florida isolate	AF049336
Squash leaf curl virus Extended host range isolate (California)	M38183
Squash leaf curl virus China isolate	AB027465
Taino tomato mottle virus Cuba isolate	AF012300
Texas pepper virus Tamuilipas (Mexico) isolate	U57457
Tomato golden mosaic virus Brazil isolate	K02029
Tomato leaf crumple virus Mexico isolate	AF101476
Tomato mottle virus Florida isolate	L14460

Table 1 (con't)

Tomato leaf curl virus Australia isolate	S53251
Tomato leaf curl virus Australia isolate-D1	AF084006
Tomato leaf curl virus Australia isolate-D2	AF084007
Tomato leaf curl virus New Delhi-1 (India) isolate	U15015/U15017
Tomato leaf curl virus New Dehli-2 (India) isolate	U15016
Tomato leaf curl virus India isolate	Z48182
Tomato leaf curl virus Bangalor-1 isolate	U38239
Tomato leaf curl virus Bangalor-2 isolate	U38239
Tomato leaf curl virus Senegal isolate	D88800
Tomato leaf curl virus India isolate	Y16421
Tomato leaf curl virus Panama isolate	Y15034
Tomato yellow leaf curl virus Israel isolate	X15656
Tomato yellow leaf curl virus Israel-2 isolate	X76319
Tomato yellow leaf curl virus Sardinia isolate	X61153
Tomato yellow leaf curl virus Sicily isolate	Z28390
Tomato yellow leaf curl virus Murcia (Spain) isolate	Z25751

Table 1 (con't)

Tomato yellow leaf curl virus Almeria (Spain) isolate	L27708
Tomato yellow leaf curl virus Portugal isolate	AF105975
Tomato yellow leaf curl virus Spain isolate (Sp7297)	AF071228
Tomato yellow leaf curl virus Spain isolate	AJ223505
Tomato yellow leaf curl virus Dominican Republic isolate	AF024715
Tomato yellow leaf curl virus Iran isolate	AJ132711
Tomato yellow leaf curl virus Shizuoka (Japan) isolate	AB014346
Tomato yellow leaf curl virus Aichi (Japan) isolate	AB014347
Tomato yellow leaf curl virus Taiwan isolate	U88692
Vigna mungo yellow mosaic virus India isolate	AJ132575

One mutant form of the AL2 gene is a double domain mutant which comprises mutations in at least two regions of the AL2 gene. The double domain mutant comprises at least one mutation in the 3' region of the AL2 gene , i.e. the region that encodes the C terminus of TrAP which extends from about amino acid 83 to about amino acid 129 of TrAP and at least one mutation in the central region of the AL2 gene, i.e., the region which encodes from about amino acid 23 to amino acid 43 of TrAP. Thus, the double domain mutant AL2 gene encodes a TrAP having at least one mutation in the acidic activation domain and at least one mutation in the cysteine-histidine domain.

Expression of an AL2 gene having a mutation in the 3' region results in a defective TrAP protein that is less able to activate transcription than a wild-type TrAP protein. The 3' region mutation is: a deletion of from 3 to 15 base pairs; or an addition of from 3 to 15 base

pairs; or a substitution of one or more base pairs in the region that encodes the C-terminus of TrAP; or combinations thereof. Preferably, the first mutation comprises a substitution of base pairs so that mutant gene encodes a mutant TrAP in which one or more amino acids in the region extending from amino acid 100-129 of the wild-type protein are substituted. More preferably, the 3' region mutation is a substitution which results in replacement of one or more of the hydrophobic amino acid residues, or one or more acidic acid residues, or combinations thereof in the C-terminus of the wild-type protein with an amino acid that does not have an acidic, hydrophobic, or aromatic side chain. Thus, it is preferred that the substituted amino acids be replaced with serine, threonine, proline, cysteine, methionine, lysine, histidine, arginine, asparagine, glutamine, glycine or alanine. Most preferably, the 3' region mutation results in replacement of the conserved isoleucine, phenylalanine, tryptophan or acidic residues within amino acids 115-129 of the wild-type protein with a small, non-charged amino acid, such as, for example, alanine or glycine. Good results in have been achieved using a mutant TGMV AL2 gene which encodes a protein having an alanine at positions 119, 123, 124, and 128 in place of the hydrophobic residues that are found at these positions in the wild-type TGMV TrAP (i.e. isoleucine 119, phenylalanine 123, tryptophan 124 and phenylalanine 128.) Good results have also been obtained using a mutant AL2 gene having an alanine at position 120 in place of an acidic aspartate.

Expression of an AL2 gene having a mutation in the central region results in a TrAP protein that is less able to bind to the cell protein SNF-1 kinase. The central region mutation is a deletion of 3 to 60 base pairs so that the TrAP encoded by the mutant gene comprises from one to 20 fewer amino acids in the central region, an addition of from 3 to 60 base pairs so that the TrAP encoded by the double domain mutant gene comprises 1 to 20 more amino acids in the central region thereof, or a substitution of one or more base pairs in the central region so that the amino acid sequence of the TrAP encoded by the double domain mutant has a sequence which is different from the sequence of the wild-type TrAP. Preferably, the second mutation comprises a deletion of from one to 10 codons which encode amino acids 33 through 43 of TrAP or, alternatively, a substitution of base pairs so that the mutant gene encodes a TrAP in which one or, preferably, more amino acids in the central region of the wild type-protein are substituted. More preferably, the double dominant mutant gene encodes a TrAP in which a plurality of cysteine residues or a combination of histidine and cysteine residues in the central region of the wild-type protein are replaced with an amino acid other than a cysteine, histidine, or methionine. It is highly preferred that the cysteine and histidine residues be replaced with glycine or alanine. Most preferably, the mutant gene encodes a

TrAP in which the histidine at position 40 is replaced with an alanine or glycine. Double domain mutant AL2 genes are used to make defective TrAP proteins that are unable to activate transcription of CP and BR1 movement protein genes and that are also unable to interact with SNF-1 kinase. Such genes can be used as research tools to study transcriptional activation in plants, .to analyze transcription factor interactions, to study viral pathogenesis, or to study plant defense responses to pathogens.

The present invention also provides mutant single domain mutant AL2 genes which have at least one mutation in the 3' region of the AL2 gene. Single domain mutant AL2 genes may also have one or more mutations in the region encoding the amino terminus of TrAP, i.e. amino acids 1 to about 22 in TrAP. Optionally, single domain mutant AL2 genes may also have one or more mutations in the region encoding the central region of TrAP, as long as such mutation or mutations are silent or involve replacement with conservative amino acids. Thus, the single domain mutant AL2 gene does not encompass the double domain mutant AL2 gene. The mutation or mutations in the 3' region of the single domain mutant AL2 gene is a deletion of from 3 to 36 base pairs, an addition of from 3 to 36 base pairs, a substitution of one or more base pairs, or combinations thereof. Preferably, the mutation comprises a substitution of base pairs so that mutant gene encodes a TrAP in which one or more amino acids in the C-terminus of the wild-type protein are substituted with a small, non-charged amino acid, such as for example alanine. More preferably, the mutant gene encodes a TrAP in which one or more of the hydrophobic amino acid residues, one or more acidic residues, or combinations thereof in the C-terminus of the wild-type protein are replaced with an alanine residue. Most preferably, the substitution mutation encodes a TrAP where the conserved isoleucine, phenylalanine, tryptophan, aspartate and glutamate residues within amino acids 115-129 of the wild-type sequence are substituted with a small, non-charged amino acid, such as for example alanine.

Single domain mutant AL2 genes are used to make defective TrAP proteins which are unable to activate transcription of the CP and BR1 movement protein genes. Such proteins, however, are able to interact with SNF-1 kinase and block pathways that employ such kinase. . Such genes can be used as research tools to study transcriptional activation in plants, .to analyze transcription factor interactions, to study viral pathogenesis, or to study plant defense responses to pathogens.

Mutations are introduced into the AL2 gene by conventional techniques such as, for example, by a combination of polymerase chain reaction (PCR) and site-directed mutagenesis. The PCR-based site-directed mutagenesis technique preferably employs a

circular plasmid or linear DNA comprising the AL2 gene from any Begomovirus, more preferably a TGMV AL2 gene. The PCR-based technique also employs oligonucleotide primers which encode the desired mutation, hereinafter referred to as the "mutagenic primer", and oligonucleotide primers that are specific for the AL2 gene and flanking sequences such that a full-length AL2 gene, or a truncated version thereof, containing the desired mutation is synthesized. The mutagenic primer comprises a first sequence of nucleotides that bind to the encoding strand of the AL2 gene immediately upstream of the point of mutation and a second sequence of nucleotides that bind to the encoding strand of the AL2 gene immediately downstream of the point of mutation.

Vectors Comprising Mutant AL2 Genes

The present invention also provides vectors comprising a double domain mutant AL2 gene and vectors comprising a single domain mutant AL2 gene. Vectors comprising mutant AL2 genes are useful for transfecting, preferably transforming plants.

The vector DNA is any DNA molecule, such as a plasmid or a bacteriaphage genome which comprises the mutant AL2 gene. The preferred vector-containing organism is a non-tumor inducing bacteria or strain of bacteria, preferably of the family Rizobiacea more preferably *Agrobacterium*, most preferably *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. Methods for transforming plants using *Agrobacterium tumefaciens* are described in Hosrsch et al (1985) *A simple and general method for transferring genes into plants*. Science 227: 1229-1231; and Rogers et al. (1986) *Production of transformed plants using Ti plasmid vectors*. Methods in Enzymology 118: 627-640.

Preferably the mutant AL2 gene, flanked by promoter and polyadenylation signals, is contained in a plasmid which is located within the bacterium. Preferably, the plasmid also contains a drug or antibiotic resistance gene which allows for positive selection of transformants in medium containing the respective drug or antibiotic. Such plasmid is introduced into the bacterium using conventional methods such as for example electroporation or cocultivation. Where the bacterium is *Agrobacterium*, it is preferred that the mutant AL2 gene be inserted into a binary plasmid which is then inserted into the *Agrobacterium* to provide an *Agrobacterium* transformant.

Methods of Making Transgenic Plants

The present invention also relates to a method of making plants that are more resistant to infection with geminiviruses, particularly Begomoviruses. The method comprises the steps of transforming a plant sample using conventional techniques with a vector comprising a double domain mutant AL2 gene, and generating a transformed plant from the transformed

plant sample. Preferably, the plant sample is obtained from a plant that is a host for Begomovirus. Plants that are susceptible to infection with a Begomovirus include, but are not limited to, beans, squash, peppers, potatoes, cassava, cotton, tobacco, kenaf, tomato, pumpkin, Arabidopsis, and cabbage.

The present invention also relates to methods of making a plant that is more susceptible to infection by Begomoviruses and Curtoviruses. The method comprises the steps of transforming a plant sample with a vector comprising a single domain mutant AL2 gene, and generating a transformed plant from the transformed plant sample. Any dicot can be made more susceptible to such infection using such method.

Conventional techniques are used for preparing transgenic plants which express the double domain mutant AL2 gene or the single domain mutant AL2 gene. Preferably, the desired mutant AL2 gene is introduced into the plant sample using an *Agrobacterium* transformant. The *Agrobacterium* transformant is cocultivated with plant cells or plant tissues. The *Agrobacterium* binds to the plant cell walls and transfers the plasmid or a portion thereof into the plant cell. Where the vector is *Agrobacterium tumefaciens*, transformation results from the transfer of a specific portion of the plasmid, referred to hereinafter as "T-DNA", into the genome of plant cells. The T-DNA is transferred and integrated into the plant genome as a discrete unit. The T-DNA contains a double domain mutant AL2 gene or a single domain mutant AL2 gene which, preferably, is flanked by a promoter and polyadenylation signals. Preferably, the T-DNA also contains a selectable marker resistance gene, such as a neomycin resistance gene. The transformed plant cells are selected by growth in medium containing the respective drug. Thereafter, transformed plants are regenerated from the cells using conventional techniques and analyzed to ensure that the transformed plant is expressing the mutant AL2 gene.

Transgenic Plants Comprising Mutant AL2 Genes.

The present invention provides transgenic plants comprising a double domain mutant AL2 gene. Such plants are resistant to infection with geminiviruses, particularly Begomoviruses As used herein, the term "resistant", means a significant reduction in geminivirus replication in the transgenic plant as compared to a non-transformed plant of the same species when such plants are inoculated with a Begomovirus. Such reduction in replication is accompanied by a significant reduction in disease symptoms in the transgenic plants. Since TrAP function is not virus specific within the Begomoviruses, a transgenic plant comprising a mutant AL2 gene of one Begomovirus is expected to be resistant to infection with all Begomoviruses.

The present invention also provides transgenic plants that are more susceptible to infection by a Begomovirus or a Curtovirus. Such plants comprise a single domain mutant AL2 gene. Examples of such plants include non-desirable plants that are employed in field studies. Following completion of the field study the transformed plants are infected with a geminiviurus from the genus Curtovirus or the genus Begomovirus. Such infection kills the plant, thereby ensuring non-propagation of the non-desirable plant. Such plants also include plants that may be used as temporary ground cover or that may be used as a source of additional soil nitrogen. Such plants may also be employed as research tools for the genetic analysis of defense pathways in plants. Specifically, transgenic plants that comprise a single domain mutant AL2 are used as model systems for identifying micro-organisms, such as for example bacteria and other viruses, whose infectivity is controlled by the SNF-1 kinase defense pathway of the plant.

Isolated Nucleic Acids Encoding the C-terminal Region of TrAP

The present invention also provides an isolated nucleic acid encoding the activation domain of TrAP, i.e. from about amino acid 100 to amino acid 129. Such nucleic acid is a useful research tool for analysis of transcriptional activation mechanisms in eukaryotic cells.

Example 1. Single Domain Mutant of an AL2 gene

A single domain mutant of the AL2 gene of TGMV encoding amino acids 1-83 of wild-type TGMV TrAP was made by PCR site-directed mutagenesis using pTGA6 as a template. pTGA6 is made by cloning the TGMV DNA A containing the AL2 gene into the *Eco*R1 site of plasmid pGEM4, which was obtained from Proem. The wild type TGMV AL2 gene and the mutant AL2 gene fragment were ligated into an two separate expression vectors which encode a GAL4 DNA binding domain (GAL4). One vector encodes a fusion protein GAL4: $TrAP_{1-129}$ which comprises the wild type TrAP protein fused the GAL4 DNA binding domain. The other vector encodes a fusion protein GAL4:$TrAP_{1-83}$ which comprises residues 1-83 of TGMV TrAP fused to the GAL4 DNA binding domain.

Example 2. Single Domain Mutant of an AL2 gene

A mutant gene which encodes a fusion protein, GAL4:$TrAP_{1-100}$, comprising amino acids 1 to 100 of TGMV TrAP, was made as described in Example 1.

Example 3. Fragment of the 3' Region of an AL2 gene

A mutant gene which encodes a fusion protein, GAL4:$TrAP_{83-114}$, comprising amino acids 83-114 of TGMV TrAP, , was made as described in Example 1.

Example 4 : Fragment of the 3' Region of an AL2 gene

A mutant gene which encodes a fusion protein, GAL4:TrAP$_{83-120}$, comprising amino acids 83-120 of TGMV TrAP, , was made as described in Example 1.

Example 5: Fragment of the 3' Region of an AL2 gene

A mutant gene which encodes a fusion protein, GAL4:TrAP$_{101-129}$, comprising amino acids 101-129 of TGMV TrAP, , was made as described in Example 1.

Example 6: Fragment of the 3' Region of an AL2 gene

A mutant gene which encodes a fusion protein, GAL4:TrAP$_{115-129}$, comprising amino acids 115-129 of TGMV TrAP, , was made as described in Example 1.

Example 7: Fragment of the 3' Region of an AL2 gene

A mutant gene which encodes a fusion protein, GAL4:TrAP$_{120-129}$, comprising amino acids 120-129 of TGMV TrAP, , was made as described in Example 1.

The ability of the fusion proteins encoded by the mutant genes of Examples 1-7 to activate transcription was assessed using an NIH3T3 cell assay system. In such assay the expression plasmid comprising the mutant form of the AL2 gene fused in frame to the GAL4 DNA binding domain is cotransfected into mouse fibroblasts (NIH3T3 cells) with an indicator plasmid consisting of the CAT reporter gene driven by a minimal promoter containing multiple copies of the GAL4 upstream activating sequence (UAS), which is specifically recognized by the GAL4 DNA binding domain. Following co-transfection, fusion proteins bind the minimal promoter, and their ability to activate transcription from this location is measured as a function of the amount of CAT present in cell extracts. CAT protein levels are quantified by ELISA using an antibody specific to CAT (Boehringer Mannheim Biochemica).

The results indicated that the fusion protein GAL4:TrAP$_{1-129}$, which comprises full length TrAP fused to the GAL4 DNA binding domain, activated transcription. Fusion proteins lacking the C-terminal acidic domain of TrAP (GAL4:TrAP$_{1-83}$ and GAL4:TrAP$_{1-100}$) or fusion proteins containing portions of the C-terminal acidic domain of TrAP (GAL4:TrAP83-114. GAL4:TrAP83-125, GAL4:TrAP83-120, GAL4:TrAP120-129) were unable to activate transcription from the minimal promoter. However, GAL4:TrAP41-129, GAL4:TrAP$_{83-129}$ GAL4:TrAP115-129 and GAL4:TrAP$_{101-129}$ were able to activate transcription. These results indicate that TrAP has an acidic activation domain which encompasses the C-terminal region of the protein. The sequence of this region is

EESIGSPQGISQLPSMDDIDDSFWENLFK. SEQ ID NO: 14. These results also indicate that the carboxy terminal 15 amino acids (115-129) of TrAP are sufficient to activate CAT expression from the indicator plasmid. Fusion proteins containing the C-terminal acidic region of TrAP but lacking amino acids 1-41 thereof were at least four times more active than fusion proteins containing full length TrAP.

Example 8 Substituted C Terminal Region of TGMV TrAP

DNA molecules encoding the C-terminal region of TGMV TrAP, i.e. either amino acids 81-129 of TGMV TrAP or amino acids 117-129, were used to determine the effect of substitutions in the C terminal region of TrAP on the ability of this domain to activate transcription. Substitutions in the nucleic acid fragment encoding the C-terminal region of TrAP were made by PCR site-directed mutagenesis using pTGA6 as a template and the primers shown in Fig. 2 One substituted fragment which has an alanine rather than an aspartic acid at position 117 was ligated into an expression plasmid containing a sequence encoding the GAL4 DNA binding domain (GAL4) to produce fusion protein, GAL4:D117-A.

Example 9. Substituted C Terminal Region of TGMV TrAP

A DNA molecule which encodes fusion protein GAL4:D118-A was made as described as above in example 8. The fusion protein encoded by this molecule comprises a C terminal region of TGMV TrAP in which the aspartic acid at position 118 is substituted with an alanine.

Example 10 Substituted C Terminal Region of TGMV TrAP

A DNA molecule which encodes fusion protein GAL4:I119-A was made as described as above in example 8. The fusion protein encoded by this molecule comprises a C terminal region of TGMV TrAP in which the isoleucine at position 11p is substituted with an alanine.

Example 11 Substituted C Terminal Region of TGMV TrAP

A DNA molecule which encodes fusion protein GAL4:D120-Awas made as described as above in example 8. The fusion protein encoded by this molecule comprises a C terminal region of TGMV TrAP in which the aspartic acid at position 120 is substituted with an alanine.

Example 12 Substituted C Terminal Region of TGMV TrAP

A DNA molecule which encodes fusion protein GAL4:D121-A was made as described as above in example 8. The fusion protein encoded by this molecule comprises a C terminal region of TGMV TrAP in which the aspartic acid at position 121 is substituted with an alanine.

Example 13 Substituted C Terminal Region of TGMV TrAP

A DNA molecule which encodes fusion protein GAL4:S122-Awas made as described as above in example 8. The fusion protein encoded by this molecule comprises a C terminal region of TGMV TrAP in which the serine at position 122 is substituted with an alanine.

Example 14 Substituted C Terminal Region of TGMV TrAP

A DNA molecule which encodes fusion protein GAL4:F123-A was made as described as above in example 9. The fusion protein encoded by this molecule comprises a C terminal region of TGMV TrAP in which the phenylalanine at position 123 is substituted with an alanine.

Example 15 Substituted C Terminal Region of TGMV TrAP

A DNA molecule which encodes fusion protein GAL4:W124-A was made as described as above in example 8. The fusion protein encoded by this molecule comprises a C terminal region of TGMV TrAP in which the tryptophan at position 124 is substituted with an alanine.

Example 16 Substituted C Terminal Region of TGMV TrAP

A DNA molecule which encodes fusion protein GAL4:E125-A was made as described as above in example 8. The fusion protein encoded by this molecule comprises a C terminal region of TGMV TrAP in which the glutamic acid at position 125 is substituted with an alanine.

Example 17 Substituted C Terminal Region of TGMV TrAP

A DNA molecule which encodes fusion protein GAL4:F128-A was made as described as above in example 8. The fusion protein encoded by this molecule comprises a C terminal region of TGMV TrAP in which the phenylalanine at position 128 is substituted with an alanine.

Example 18 Substituted C Terminal Region of TGMV TrAP

A DNA molecule which encodes fusion protein GAL4:D117,118-Awas made as described as above in example 8. The fusion protein encoded by this molecule comprises a C terminal region of TGMV TrAP in which the aspartates at positions 117 and 118 are each substituted with an alanine.

Example 19 Substituted C Terminal Region of TGMV TrAP

A DNA molecule which encodes fusion protein GAL4:D120,121-A was made as described as above in example 8. The fusion protein encoded by this molecule comprises a C terminal region of TGMV TrAP in which the aspartates at position 120 and 121 are each substituted with an alanine.

The ability of the substituted C terminal fragments of TrAP to activate transcription was assessed using an NIH3T3 cell assay system. The results showed that substitution of

hydrophobic residues isoleucine 119, phenylalanine 123, tryptophan 124 and phenylalanine 128 resulted in nearly complete loss of activity, indicating that each of these residues is critical for activation. With the exception of aspartate 120 which also appears to be critical for activation, substitution of the acidic amino acids (specifically, aspartate 177. aspartate 118, aspartate 121 and glutamate 125) reduced activity to a lesser extent, i.e., to roughly 50% of the reduction observed when the hydrophobic residues were substituted. Multiple substitutions of acidic residues, such as for example substitutions at aspartate 117 and aspartate 118, reduced activity to a greater extent than single substitutions. Thus, mutant AL2 genes comprising such substitutions are unable to activate transcription.

Example 20: Truncated AL2 Genes Comprising a Substitution in the Region Encoding the Cysteine-Histidine Rich Region of TrAP

The yeast two-hybrid system was used to identify proteins that bind to the amino terminus and central region of TrAP. Specifically, the system was used to screen an Aribidopsis cDNA library for such proteins. A truncated form of TrAP, TrAP1-83 was used as bait because full-length TrAP activates transcription by itself. From this screen, a cDNA encoding a homologue of yeast SNF-1 kinase was isolated. Two-hybrid analysis showed that TrAP specifically interacts with the Arabidopsis SNF-1 kinase and yeast SNF-1 kinase, but not with other TGMV proteins or other cellular negative control proteins.

Using the two hybrid method with defined regions of TrAP as bait, the SNF-1 interaction domain of TrAP was located to amino acids 23-43. This region contains the first four conserved cysteine and histidine residues (C-X1-C-X4-H-X2-C) of the central region.

Similar studies indicated that the L2 protein of the Curtovirus BCTV also interacts with SNF-1 kinase. TRAP and L2 protein are homologous in the CCHC motif indicated above. The cysteine and histidine residues comprising this motif are found at positions 33, 35, 40, and 43 in TrAP. PCR site-directed mutagenesis was used to replace the cysteine and histidine residues at these positions with alanine. The mutagenic primers used were:

C33: 5'-gacctgaacgctggctgttcc-3'; SEQ ID NO: 15

C35: 5'-gaactgtggcgcttccatatac-3'; SEQ ID NO: 16

H40: 5'-ccatatacattgccatcgactgc-3'; SEQ ID NO: 17

C43: 5'-cacatcgacgccagaaacaatgg-3', SEQ ID NO: 18.

Mutations were made using these primers and the Muta-gene in vitro Mutagenesis Kit from Bio-Rad. Template DNA used in the mutagenesis consisted of the TGMV AL2 open reading frame cloned into M13mp18RF DNA. M13 phage containing the desired mutations were confirmed by sequencing. Single-stranded DNA was then used as template for PCR

reactions which amplified the mutant AL2 ORF. Primers used in these reactions were as follows:

5' primer: 5'-gcgagatctatgcgaaaattcgtcttcc-3'; SEQ ID NO: 19

3' primer: 5'-cgcgagctcctattaaataagttctccca-3': SEQ ID NO: 20

The resulting PCR products were cloned into pBI221 (Clonetech), downstream of the 35S promoter, for subsequent expression in plant cells.

In addition, the mutant AL2 genes were amplified by PCR using as 5' primer: 5' gcgggcgccatgcgaaattcgtcttcc-3', SEQ ID NO: 21 and as 3' primer: 5' cgcgagctcctatttaaataagttctccca-3', SEQ ID NO: 22. The resulting PCR products were then digested with BamH1 and Ehe1 and cloned into pAS2 and pACT2 for testing in the yeast two hybrid system.

The yeast two-hybrid results showed that TrAP H40A, which comprises an alanine at position 40 rather than a histidine lost the ability to interact with SNF-1 kinase. TrAP C33A, and C43A were still able to interact with SNF-1 kinase.

Example 21: Transgenic Plants Comprising a Single Domain Mutant AL2 Gene

Transgenic N. benthamiana plants comprising single domain mutant AL2 genes were made using an A. tumefaciens transformant that was prepared using a binary system. The transgenic plants contained a single domain mutant AL2 gene which encoded one of the following TrAP proteins: TrAP F123A, TrAP W124A, TrAP F128A, TrAP 1-115, TrAP 1-120, TrAP- 1-125 and, TrAP1-83. Transgenes were constructed by PCR. The 5' primer employed in all cases was 5'GCGAGATCTATGCGAAATTCGTCTTCC-3', SEQ ID NO: 23. which is complementary to a sequence at the 5' end of the TGMV AL2 gene. Template DNA employed in the PCR reaction was pTGA6.

A. Materials and Methods Used to Prepare the A. tumefaciens Transformant

The binary system consists of two components: 1) a disarmed A. tumefaciens Ti plasmid, which provides functions required for excision of the T-DNA from the Ti plasmid, and for its transfer and integration into the plant genome and 2) a second, smaller binary plasmid vector of about 6 - 10 kb that contains the T-DNA to be transferred, i.e. the mutant AL2 gene and a drug resistance gene.

Cultures of E. coli harboring the binary vectors were grown in LB broth containing 50 μg/ml spectinomycin. The binary vectors contained the mutant AL2 genes shown in Table III below and a selectable marker. Most of the binary vectors also included a streptomycin/spectinomycin resistance gene (outside the T-DNA) for selection in E. coli and A. tumefaciens. Binary vectors used included pMON530 prepared as described in (Rogers et

al. (1987) *Improved vectors for plant transformation: Expression cassette vectors and new selectable markers.* Methods in Enzymology 153: 253-277). Alternatively, the binary vectors used were proprietary vectors obtained from Agritope, Inc. pMON521 contains the 35S promoter. The three different Agritope vectors used are designed to express the transgene at low, moderate, and high levels.

Non-transformed A. tumefaciens containing the resident, disarmed Ti plasmid was obtained from Monsanto. Cultures of the non-transformed A. tumefaciens were grown overnight in LB broth containing 25 μg/ml chloramphenicol and 50 μg/ml kanamycin. Cultures of E. coli containing the mobilization plasmid (e.g. pRK2013) was grown overnight in LB broth containing 50 μg/ml kanamycin. Cultures of E. coli containing the binary plasmid were grown overnight in LB broth containing 50 μg/ml spectinomycin.

1-2 drops of each overnight culture were mixed and streaked on an LB plate containing no selection marker. The plates were incubated at 28°C until colonies appeared, usually for about 6 days. Then a small amount of culture was recovered from the LB plate and streaked on LB plates containing a selection markers or antibiotics which corresponded to the resistance markers of the A. tumefaciens recipient containing the disarmed Ti plasmid and the binary vector to select A. tumefaciens transformants.

The plates were incubate at 28°C until colonies were detected on the plates. Several individual colonies were recovered from each plate and inoculated into LB broth containing the same antibiotics as the plate and incubate at 28°C with shaking.

B. Transforming plant samples

Plasmid DNA was isolated from A. tumefaciens transformants according to the method of Dhaese et al. (1979) Nucleic Acids Research 7: 1837 was used to verify the presence and integrity of mutant AL2 genes in T-DNA by Southern blot analysis or PCR. A. tumefaciens transformants were also used to transform leaf discs from Nicotiana benthamiana plants. For petunia and tobacco leaf discs are also used. For tomato, cotyledon pieces are used. For Arabidopsis thaliana, sterile root pieces are used. Prior to transformation, the plant tissues were sterilized in a solution of 20% chlorox, 0.5% Tween 20 for 15 min.

The leaf discs were placed upside down on MS104 plates and preincubated for 48 hours at room temperature in continuous light to increase the transformation efficiency Thereafter, the sterilized leaf discs were soaked in a liquid culture of an A. tumefaciens transformant. This is done by placing 10-20 discs in a sterilin tube (with a loose cap) and adding 1 ml of an overnight culture. The discs were then removed, blotted dry with sterile

filter paper, and placed upside down on an MS104 plate with no selection. After 48 hr on the non-selective MS104 media at room temperature, the discs were transferred to MS104 plates containing selection medium (750 μg/ml carbenicillin to kill the Agrobacterium and 300 μg/ml kanamycin to select for the desired T-DNA marker). After about 1-2 weeks, the discs form callus around the edges of the disc. This is followed by the appearance of shoots.

Shoots were removed at regular intervals from the callus and transferred to rooting media (MSO plates containing the antibiotics present in the MS104 plates). Shoots with roots were transferred to sterile soil in pots and covered with clear plastic to retain humidity. After 2-4 days, plastic can be removed and transgenic plants treated as normal plats.

Transformed leaf discs are harvested and analyzed for presence of the transgene, mRNA and protein expression after 2-6 days. Alternatively transformed regenerants are obtained and analyzed in the same manner. DNA, RNA or protein is isolated from the leaf discs or regenerated plants by conventional methods. The presence of an integrated AL2 gene in the genome of the plant is examined by restriction endonuclease digestion followed by Southern blot analysis, or by PCR using primers designed to recognize T-DNA border sequences Expression of RNA encoding the mutant AL2 gene is examined by Northern blot analysis or by RT-PCR. Expression of mutant TrAP is examined by Western blotting.

Transgenic lines are established by selfing transformed plants to homozygosity using conventional techniques.

C. Susceptibility of the transgenic plants to infection with Begomoviruses

Transgenic plants made as described above and comprising the mutant AL2 genes shown in Table III were tested by challenge inoculation with TGMV and BCTV. In initial tests, viruses were delivered to plants by the agroinoculation procedure described in Elmer et al. (1988) *Agrobacterium-mediated inoculation of plants with tomato golden mosaic virus DNAS.* Plant Mol. Biol. 10:225-234. In subsequent tests with TGMV, the virus was mechanically inoculated. Specifically, pUC based plasmid DNAs containing tandem repeats of TGMV DNA A and TGMV DNA B were mixed and mechanically inoculated into plant leaves as a DNA solution with the aid of celite abrasive.

One mutant gene used for transformation encoded a truncated form of TrAP, i.e., TrAP1-83, which was under control of the strong and constitutive 35S promoter from cauliflower mosaic virus. Transgenic plants comprising this mutant gene exhibited a normal phenotype until inoculated with TGMV and BCTV. Following inoculation these transgenic plants showed an enhanced susceptibility phenotype, characterized by a much reduced latent period and by infection at much lower inoculum doses that non-transformed plants of the

same species. Plants transfected with a full-length wild-type AL2 gene died prior to inoculation. It is believed that the enhanced susceptibility of the transgenic plants is due to interaction of the modified TrAP with SNF1 kinase.

Another mutant gene used for transformation encode a full-length TrAP protein in which the tryptophan at position 124 in the wild-type sequence is replaced with an alanine. The transgenic plant comprising this single domain mutant AL2 gene also exhibited enhanced susceptibility to infection.

Example 22 A double domain mutant AL2 gene encoding a mutant TrAP having an amino acid substitution in the central Region (H40A) and an amino acid substitution in the C terminal Region (F123A).

The double domain mutant was made in two steps. In the first step, a single domain mutant AL2 gene comprising a variant sequence which encodes a mutant TrAP having an alanine at position 40 rather than the histidine which is found at position 40 in the wild-trap TrAP was made. The single domain mutant was made using the H40 mutagenic primer, SEQ ID NO 17, of example 21 and the Muta-Gene in vitro mutagenesis kit from Bio-Rad. Template DNA used in the mutagenesis consisted of the TGMV AL2 open reading frame cloned into M13mp18RF DNA. Using the Muta-Gene system, the AL2 gene cloned in the vector was first transformed into a dut-/ung- E. coli strain and single-stranded phage DNA containing uracil was isolated. The phage DNA was annealed with the mutagenic primer (in this case H40A, SEQ ID NO: 17), and the second strand was synthesized with T7 DNA polymerase and T7 DNA ligase. The double-stranded DNA was transformed into an ung+ E. coli strain (which degrades uracil-containing DNA); consequently the parental strand was not replicated and the strand synthesized using the mutagenic primer was preferentially amplified. M13 phage containing the desired H40A mutation were selected and confirmed by sequencing. In the second step a second mutation in the mutant H40A mutant AL2 gene was carried out by PCR amplification of the mutant H40 AL2 gene, using a wild type 5' primer (618A-5'; Figure 2, SEQ ID NO: 26) and a 3' primer (620-3'; Figure 2, SEQ ID NO: 31) designed to create a full-length AL2 gene containing the F123A substitution. Thus, the double mutant AL2 gene encodes a full-length mutant TrAP having an alanine at position 40 in the amino acid sequence rather than a histidine and an alanine at position 123 rather than a phenylalanine

Example 23. A double domain mutant AL2 gene encoding a mutant TrAP having a deletion within the central (delta 33-43) region and a deletion within the C terminal region (delta 115-129).

The double domain mutant AL2 gene comprising a variant sequence which encodes a TrAP protein lacking amino acids 33 through 43 of the central region, and lacking amino acids 115 through 129 of the C terminal region were made by bidirectional PCR. Plasmid pTGA6, which contains a single copy of TGMV A DNA, was used as template. In one reaction, a wt 5' primer (618A; Figure 2, SEQ ID NO: 26) was used in combination with 3' primer (CCHC-1; GTTCAGGTCAATTCGTCGCCT). This primer combination permits amplification of the AL2 sequence encoding amino acids 1 through 32. In a second reaction, CCHC-2 (AGAAACAATGGATTCACGCAC) was used as 5' primer and 679-3', SEQ ID NO: 37 (Figure 2) was used as 3' primer. This primer combination permits amplification of the AL2 sequence encoding amino acids 44 through 114. Following amplification, the products of the two reactions were ligated together with a plasmid cloning vector (pUC) and transformed into E. coli. Plasmids containing the desired double domain mutant AL2 gene, encoding amino acids 1-32 and 44-114, were selected. Thus, the double domain mutant AL2 gene encodes a mutant TrAP which lacks amino acids 33 through 43 and amino acids 115 through 129 of the wild-type TrAP.

Example 24: Transgenic Plants Comprising the Double Domain Mutant AL2 Genes of Example 22 and 23

The mutant AL2 genes of Examples 22 and 23 are incorporated into an Agrobacterium vector as described in Example 21 and used to prepare transgenic plants as described in Example 21.

CLAIMS

What is claimed is:

1. An isolated, single domain recombinant AL2 gene encoding a modified transcription activator protein, said recombinant AL2 gene comprising a mutation in the region which encodes from about amino acid 83 to about amino acid 129 of said corresponding wild-type transcription activator protein.
2. The recombinant AL2 gene of claim 1 wherein said mutation is in the region which encodes from about amino acid 115 to about amino acid 129 of said wild-type transcription activator protein.
3. The recombinant AL2 gene of claim 1 wherein said mutation is a deletion and the modified transcription activator protein encoded by said recombinant gene has from one to 29 fewer amino acids than the corresponding wild-type transcription activator protein.
4. The recombinant AL2 gene of claim 1 wherein said mutation is a substitution of base pairs and said mutant AL2 gene encodes a modified transcription activator protein having an amino acid selected from the group consisting of serine, threonine, proline, cysteine, methionine, lysine, histidine, arginine, asparagine, glutamine, glycine and alanine in place of a hydrophobic amino acid in the C terminal region of the wild-type transcription activator protein.
5. The recombinant AL2 gene of claim 1 wherein said mutation is a substitution of base pairs and said mutant AL2 gene encodes a modified transcription activator protein having an amino acid selected from the group consisting of serine, threonine, proline, cysteine, methionine, lysine, histidine, arginine, asparagine, glutamine, glycine and alanine in place of an acidic amino acid in the C-terminal region of in the wild-type transcription activator protein.
6. The mutant AL2 gene of claim 1 wherein said mutation is a substitution of base pairs and the modified transcription activator protein encoded by the mutant AL2 gene comprises an alanine or glycine at a position selected from the group consisting of position 119, position 120, position 123, position 124, position 128, and combinations thereof.
7. An isolated, double domain recombinant AL2 gene encoding a modified transcription activator protein, said recombinant AL2 gene comprising a mutation in the region which encodes from about amino acid 83 to about amino acid 129 of said transcription activator protein and a second mutation in the region which encodes from about amino acid 23 to about amino acid 43 of said transcription activator protein.

8. The recombinant AL2 gene of claim 7 wherein said first mutation is in the region which encodes from about amino acid 115 to about amino acid 129 of said transcription activator protein.

9. The recombinant AL2 gene of claim 7 wherein said first mutation is a deletion and said modified transcription activator protein encoded by said mutant gene has from one to 20 fewer amino acids than the corresponding wild-type transcription activator protein.

10. The recombinant AL2 gene of claim 7 wherein said first mutation is a substitution of base pairs and said recombinant AL2 gene encodes a modified transcription activator protein having an amino acid selected from the serine, threonine, proline, cysteine, methionine, lysine, histidine, arginine, asparagine, glutamine, glycine and alanine in place of a hydrophobic amino acid that is found in the C terminal region of the corresponding wild-type transcription activator protein.

11. The recombinant AL2 gene of claim 7 wherein said mutation is a substitution of base pairs and said recombinant AL2 gene encodes a transcription activator protein having an amino acid selected from the serine, threonine, proline, cysteine, methionine, lysine, histidine, arginine, asparagine, glutamine, glycine and alanine in place of an acidic amino acid in the corresponding wild-type transcription activator protein.

12. The recombinant AL2 gene of claim 7 wherein said second mutation is a deletion and said modified transcription activator protein encoded by said recombinant gene has from one to 20 fewer amino acids than the corresponding wild-type transcription activator protein.

13. The recombinant AL2 gene of claim 7 wherein said second mutation is a substitution of base pairs and said recombinant AL2 gene encodes a modified transcription activator protein in which a plurality of the cyteine residues located in the central region of the corresponding wild-type transcription activator protein are substituted.

14. The recombinant AL2 gene of claim 7 wherein said second mutation is a substitution of base pairs and said recombinant AL2 gene encodes a modified transcription activator protein which comprises an alanine or glycine at position 40.

15. A vector comprising the mutant AL2 gene of claim 1.

16. The vector of claim 15 wherein said vector is an Agrobacterium.

17. A vector comprising the mutant AL2 gene of claim 7.

18. The vector of claim 17 wherein said vector is an Agrobacterium.

19. A transgenic plant comprising the single domain recombinant AL2 gene of claim 1.

20. A transgenic plant comprising the double domain recombinant AL2 gene of claim 7

21. A method for preparing a plant that is more susceptible to infection with Begomoviruses or Curtoviruses, said method comprising:

a) providing a sample from a plant which is a host for a Begomovirus or a Curtovirus; b) transforming said sample with the vector comprising a single domain recombinant AL2 gene of claim 1

c) generating a plant from said transformed sample of step (b).

22. The method of claim 21 wherein said vector is an Agrobacterium

23. A method of preparing a transgenic plant, comprising

a) providing a sample from a plant which is a host for a Begomovirus;

b) transforming said sample with a vector comprising a double domain recombinant AL2 gene of claim 7; and

c) generating a plant from said transformed sample of step (b).

24. An isolated nucleic acid encoding the transcription activation domain of the transcription activator protein of TGMV, said nucleic acid comprising the sequence EESIGSPQGISQLPSMDDIDDSFWENLFK, SEQ ID NO: 14.

Fig. 1

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TGMV       MRNSSSSTPPSIKAQHRAAKRRAIRRRRIDLNCGCSIYIHIDCRN--NGF   Seq. ID No.: 1
AbMV-HI    MRSSSPSHPPSIKKAHRQAKRRAIRRRRIDLQCGCSIYFHIDCTG--HGF   Seq. ID No.: 2
AbMV-WI    MRSSSPSQPPSIKKAHRQAKRRAIRRRRIDLQCGCSIYFHIDCTG--HGF   Seq. ID No.: 3
BDMV       MQSSSLSTPPSIKKAHRQAKRRAIRRRRIDLECGCSIYIHIGCTG--HGF   Seq. ID No.: 4
BGMV-DR    MRSSSPSQPPSIKAQHRIAKRKAIRRRRIDLNCGCSIFYHIKCAD--HGF   Seq. ID No.: 5
BGMV-Ga    MRSSSPSQPPSIKAQHRIAKRRAIRRRRIDLNCGCSIFYHIKCAD--HGF   Seq. ID No.: 6
BGMV-J     MRSSSPSQPPSIKAQHRIAKHKAIRRRRIDLNCGCSIFYHIKCAD--HGF   Seq. ID No.: 7
CabLCV     MQNSSLLKPPSIKAQHKIAKRRAVRRRRIDLNCGCSIFLHINCAD--NGF   Seq. ID No.: 8
PYMV       MRSSSPSQPPSIKKAHRQAKKRAIRRRRIDLDCGCSIYFHIDCAG--HGF   Seq. ID No.: 9
SGMV       MRSSSPSTPPSIKKTHRQAKKRSVRRRRIDLECGCSIYFHIGCTG--HGF   Seq. ID No.: 10
SqLCV-E    MPNSSSSKVPSIKAQHRIAKKRAVRRRRIDLDCGCSIYIHINCAKDGNGF   Seq. ID No.: 11
TcMV       MRSSSPSQPPSIKRAHRQAKKRAIRRRRVDLQCGCSIYFHLGCAG--HGF   Seq. ID No.: 12
TPV        MLNSSSSTLPSIKAQHRIAKKRPIRRRRIDLNCGCSIFLHINCAN--NGF   Seq. ID No.: 13
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TGMV       THRGTYHCASSREWRLYLGDNKSPLFQDNQRRGSPLHQHQDIPLTNQVQP   Seq. ID No.: 1
AbMV-HI    THRGTHHCTSGGEWRVYLGDKKSPVFQDVQSRRTIIHQNENIPCTNTVQP   Seq. ID No.: 2
AbMV-WI    THRGIHHCTSGGEWRVYLGDSKSPVFQDIQSRGPAIHQNEDIPCTNTVQP   Seq. ID No.: 3
BDMV       THRGAHHCTSSREWRVYLGDRKSPLFQDIQSRGTVVHQQQGIQRTDTVQP   Seq. ID No.: 4
BGMV-DR    THRGEHHCASGREFRFYLGGTKSPLFQDHAGGRSSIHTDKDIPHPSQVQS   Seq. ID No.: 5
BGMV-Ga    THRGEHHCASGREFRFYLGGTKSPLFQDNAGGRSSIHTDKDIPHPNQVQS   Seq. ID No.: 6
BGMV-J     THRGEHHCASGREFRFYLGGTKSPLFQDHAGGRSSIHTDKDIPHPSQVQS   Seq. ID No.: 7
CabLCV     THRGEHHCSSGREFRFYLGGSKSPIFQDTTRRGPVVHQNQDLPHPSPVQP   Seq. ID No.: 8
PYMV       THRGAHHCTSGREWRVYLGDRKSPLFQDKPSRGHAIHQDQDIQRPNPVQP   Seq. ID No.: 9
SGMV       THRGTHHCTSFREWRLYLGDRKSPLFQDIQGRGSTVHEHQDIQYPNPVQP   Seq. ID No.: 10
SqLCV-E    THMGRHHCASGREFRFYLGGSKSPLFQDVQRGGSTLHAHKDIPHTNPVQP   Seq. ID No.: 11
TcMV       THRGTHHCTSGGEWRVYLGARKSPLFQDTQSRGPTVYQNEGIPRTDTVQP   Seq. ID No.: 12
TPV        THRGEHHCASGREFRFYLGGSKSPIFQDTARGGPVVHQNQDIPHRSPVQP   Seq. ID No.: 13
           ** *  ** *  * * ***  *** ***                    **
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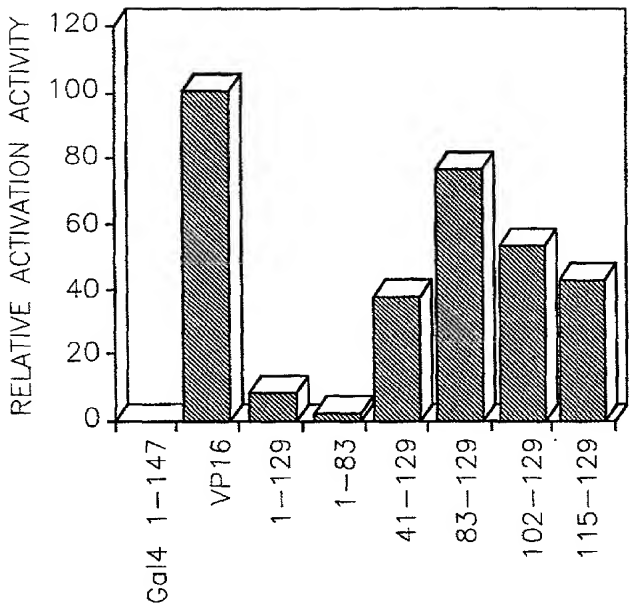
TGMV       QPEESIGSPQGISQLPSMDDIDDSFWENLFK   Seq. ID No.: 1
AbMV-HI    QPEESVASPQSLPELPSLDDIDESFWVNLFS   Seq. ID No.: 2
AbMV-WI    QPEESVASPQSLPELPSLDDFDDSFWVNLFK   Seq. ID No.: 3
BDMV       QPEESVASPQSLPELPSLDDIDDSFWVDLFK   Seq. ID No.: 4
BGMV-DR    QPQESTGSPQSIPELPSLDDIDSSFWDDIFK   Seq. ID No.: 5
BGMV-Ga    QPQESTGSPQSIPELPSLDDIDSSFWDDIFK   Seq. ID No.: 6
BGMV-J     QPQESTGSPQSIPELPSLDDIDSSFWDDIFK   Seq. ID No.: 7
CabLCV     QPTESIGSPQSLLQLPSLDDFDESFWADIFK   Seq. ID No.: 8
PYMV       QPQESIGSPQSIPELPSLDDIDDSFWVELPS   Seq. ID No.: 9
SGMV       QPEESVASPQSVPELPSLDDIPDSFWVDLFK   Seq. ID No.: 10
SqLCV-E    QPEESTKSSQSVPELPSLDGIDSSFWDDIFE   Seq. ID No.: 11
TcMV       QPEESVASPQSLPELPSLDDVDDSFWINLFS   Seq. ID No.: 12
TPV        QPTSSISSPQGIPPFPSLDDFDDSFWDDIFK   Seq. ID No.: 13
           **  *  * *     ** *    ***   *
```

Fig. 2

clone #	mutation description	5' primer	
pTGA618	81-129 wt	gcggaattccctctacaccaacaccag	Seq. ID No.: 24
pTGA618A	1-129 wt	cgcgaattcatgcgaaattcgtcttcc	Seq. ID No.: 26
pTGA619	81-129 S122A	gcggaattccctctacaccaacaccag	Seq. ID No.: 28
pTGA620	81-129 F123A	gcggaattccctctacaccaacaccag	Seq. ID No.: 30
pTGA621	81-129 W124A	gcggaattccctctacaccaacaccag	Seq. ID No.: 32
pTGA622	81-129 F128A	gcggaattccctctacaccaacaccag	Seq. ID No.: 34
pTGA679	81-114 wt	gcggaattccctctacaccaacaccag	Seq. ID No.: 36
pTGA680	81-120 wt	gcggaattccctctacaccaacaccag	Seq. ID No.: 38
pTGA681	81-125 wt	gcggaattccctctacaccaacaccag	Seq. ID No.: 40
pTGA682	81-129 E125A	gcggaattccctctacaccaacaccag	Seq. ID No.: 42
pTGA713	101-129 wt	cgcgaattcgaggagagcattggatctcca	Seq. ID No.: 44
pTGA714	114-129 wt	cgcgaattcagtatggacgacatcgac	Seq. ID No.: 46
pTGA715	114-129 I119A	cgcgaattcagtatggacgacgccgacgac	Seq. ID No.: 48
pTGA716	103-129 wt	cgcgaattcagcattggatctccacaaggc	Seq. ID No.: 50
pTGA717	81-129 K129A	gcggaattccctctacaccaacaccag	Seq. ID No.: 52
pTGA718	81-129 L127A	gcggaattccctctacaccaacaccag	Seq. ID No.: 54
pTGA719	81-129 N126A	gcggaattccctctacaccaacaccag	Seq. ID No.: 56
pTGA720	117-129 D117A	cgcgaattcgccgacatcgacgacagc	Seq. ID No.: 58
pTGA721	117-129 D118A	cgcgaattcgacgccatcgacgacagc	Seq. ID No.: 60
pTGA722	117-129 D117A, D118A	cgcgaattcgccgccatcgacgacagc	Seq. ID No.: 62
pTGA723	120-129 wt	cgcgaattcgacgacagcttcgggag	Seq. ID No.: 64
pTGA724	117-129 D120A	cgcgaattcgacgacatcgccgacagcttc	Seq. ID No.: 66
pTGA725	117-129 D121A	cgcgaattcgacgacatcgacgccagcttc	Seq. ID No.: 68
pTGA726	117-129 D120A, D121A	cgcgaattcgacgacatcgccgccagcttc	Seq. ID No.: 70
pTGA727	41-129 wt	ggcgaattcatggactgcagaaacaatgga	Seq. ID No.: 72
pTGA728	10-129 wt	gcggaattctctatcaaagctcaacac	Seq. ID No.: 74

Fig. 2 (con't)

clone #	mutation description	3' primer	
PTGA618	81-129 wt	cgcgagctcctatttaaataagttctccca	Seq. ID No.: 25
PTGA618A	1-129 wt	cgcgagctcctatttaaataagttctccca	Seq. ID No.: 27
PTGA619	81-129 S122A	cgcgagctcctatttaaataagttctcccagaaggcgtcgtcgat	Seq. ID No.: 29
PTGA620	81-129 F123A	cgcgagctcctatttaaataagttctcccaggcgctgtcgtc	Seq. ID No.: 31
PTGA621	81-129 W124A	cgcgagctcctatttaaataagttctccgcgaagctgtc	Seq. ID No.: 33
PTGA622	81-129 F128A	cgcgagctcctatttagctaagttctc	Seq. ID No.: 35
PTGA679	81-114 wt	gcggagctcctaaagttgagaaatgcc	Seq. ID No.: 37
PTGA680	81-120 wt	gcggagctcctagatgtcgtccatact	Seq. ID No.: 39
PTGA681	81-125 wt	gcggagctcctaccagaagctgtcgtc	Seq. ID No.: 41
PTGA682	81-129 E125A	gcggagctcctatttaaataagtttgcccagaa	Seq. ID No.: 43
PTGA713	101-129 wt	cgcgagctccctctaaccctgtgtatgcg	Seq. ID No.: 45
PTGA714	114-129 wt	cgcgagctccctctaaccctgtgtatgcg	Seq. ID No.: 47
PTGA715	114-129 I119A	cgcgagctccctctaaccctgtgtatgcg	Seq. ID No.: 49
PTGA716	103-129 wt	cgcgagctccctctaaccctgtgtatgcg	Seq. ID No.: 51
PTGA717	81-129 K129A	cgcgagctcctatgcaaataagttctc	Seq. ID No.: 53
PTGA718	81-129 L127A	cgcgagctcctatttaaatgcgttctc	Seq. ID No.: 55
PTGA719	81-129 N126A	cgcgagctcctatttaaataaggcctccca	Seq. ID No.: 57
PTGA720	117-129 D117A	cgcgagctccctctaaccctgtgtatgcg	Seq. ID No.: 59
PTGA721	117-129 D118A	cgcgagctccctctaaccctgtgtatgcg	Seq. ID No.: 61
PTGA722	117-129 D117A, D118A	cgcgagctccctctaaccctgtgtatgcg	Seq. ID No.: 63
PTGA723	120-129 wt	cgcgagctccctctaaccctgtgtatgcg	Seq. ID No.: 65
PTGA724	117-129 D120A	cgcgagctccctctaaccctgtgtatgcg	Seq. ID No.: 67
PTGA725	117-129 D121A	cgcgagctccctctaaccctgtgtatgcg	Seq. ID No.: 69
PTGA726	117-129 D120A, D121A	cgcgagctccctctaaccctgtgtatgcg	Seq. ID No.: 71
PTGA727	41-129 wt	cgcgagctcctatttaaataagttctccca	Seq. ID No.: 73
PTGA728	10-129 wt	cgcgagctcctatttaaataagttctccca	Seq. ID No.: 75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1–83
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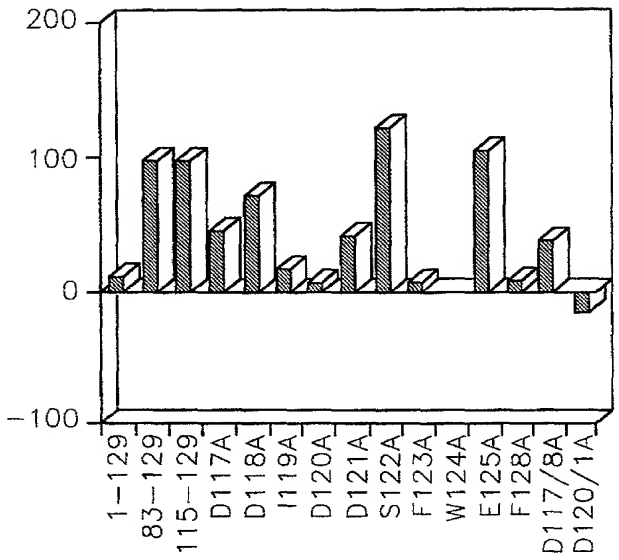
83–129
102–129
115–129

Fig.3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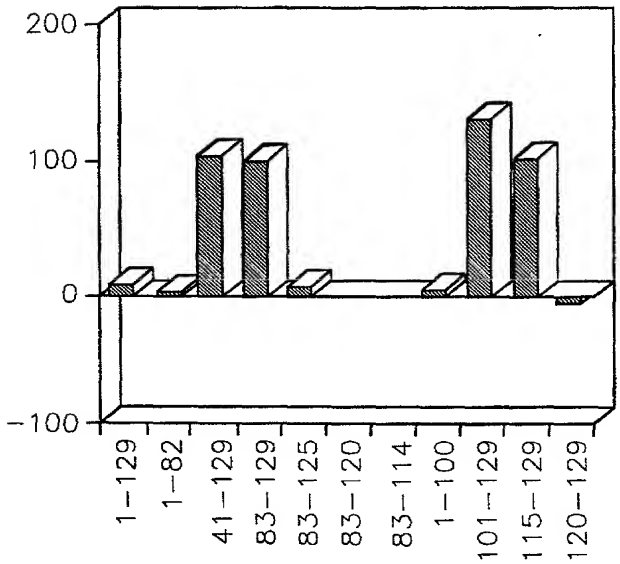
D120/1A

Fig.4A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83−114
1−100
101−129
115−129
120−129

Fig.4B

DECLARATION
AND POWER OF ATTORNEY
ORIGINAL APPLICATION

As below named inventors, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

MUTANT FORMS OF THE AL2 GENE OF GEMINIVIRUSES

the specification of which

☐ is attached hereto,
☒ was filed on June 5, 1998, as Application Serial No. 09/092,705.
☐ and was amended on _____.
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, code of Federal Regulations, §1.56(a).

I hereby appoint the following attorney(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made hereon of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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